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14. ABSTRACT

Amplification of 8p11-12 occurs in approximately 15% of human breast cancer (HBC), and this region of amplification is significantly associated with disease-specific survival and distant recurrence in breast cancer patients. Earlier, we used genomic analysis of copy number and gene expression to perform a detailed analysis of the 8p11-12 amplicon to identify candidate oncogenes in breast cancer. We identified Wolf-Hirschhorn syndrome candidate 1-like 1 (WHSC1L1) as a candidate oncogene based on statistical analysis of copy number increase and overexpression. In this study, we demonstrated that WHSC1L1 acts as a transforming gene: stable WHSC1L1 overexpression in nontumorigenic MCF10A cells induces transformed phenotypes, whereas WHSC1L1 knockdown in 8p12 amplified, ER-positive breast cancer cells inhibits proliferation in vitro. We also found that overexpression of WHSC1L1 likely induces the acquisition of stem cell-like properties in vitro. WHSC1L1 family proteins have recently been shown to bind methylated histones, specifically H3K36 methylation marks. We determined global methylation levels in a panel of breast cancer cell lines, including WHSC1L1-amplified SUM-44 and SUM-52 lines. These breast cancer cell lines with defined histone methylation levels will provide a useful model to further study how WHSC1L1 contributes to transformation through the alteration of epigenetic histone marks in a subset of aggressive breast cancers.

15. SUBJECT TERMS
Gene amplification, PWWP-domain, histone modification

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Introduction

Amplification of 8p11-12 occurs in approximately 15% of human breast cancer (HBC), and this region of amplification is significantly associated with disease-specific survival and distant recurrence in breast cancer patients (1-5). Earlier, we used genomic analysis of copy number and gene expression to perform a detailed analysis of the 8p11-12 amplicon to identify candidate oncogenes in breast cancer (4). We identified Wolf-Hirschhorn syndrome candidate 1-like 1 (WHSC1L1) as a candidate oncogene based on statistical analysis of copy number increase and overexpression (4). The WHSC1L1 gene encodes a PWWP domain protein that regulates gene transcription and differentiated function of cells through regulation of histone methylation (6, 7). In this proposal, we hypothesize that WHSC1L1 is the major driving oncogene in the 8p11 amplicon that is found in aggressive forms of ER positive, luminal breast cancers. Furthermore, we hypothesize that genetic deregulation of WHSC1L1 induces alterations in the epigenetic histone code resulting in the acquisition of cancer stem cell phenotypes. Based on this hypothesis, we predict that WHSC1L1 will be a good therapeutic target in breast cancer, particularly for those ER positive breast cancers that are, or become, refractory to endocrine therapy.

Body

1. Specific Aims

This project consists of 3 specific aims:
Aim 1: To investigate the molecular mechanism, including the structural details, of WHSC1L1 that is involved in the transforming function through the alteration of the epigenetic histone code in human breast cancer cells.
Aim 2: To determine whether the histone modulation function of WHSC1L1 is linked to cancer stem cell phenotypes.
Aim 3: To examine the potential of WHSC1L1 as a therapeutic target in aggressive, ER-positive breast cancers that harbor the 8p11 amplicon.

2. Studies and Results

***Task 1*. To investigate the molecular mechanism, including the structural details, of WHSC1L1 that is involved in transforming function through the alteration of the epigenetic histone code in human breast cancer cells**.

In our previous annual reports, we have stated that we identified 21 candidate oncogenes within the 8p11-12 amplicon in breast cancer based on statistical analysis of copy number increase and gene overexpression. Using gain- and loss-of- function approaches, we found that WHSC1L1 is the most potently transforming oncogene we tested from the 8p11-12 region. Expression of the WHSC1L1 gene results in two alternatively spliced variants, a long isoform and a short isoform, that are derived from alternative splicing of exon 10. The WHSC1L1 long isoform encodes a 1437 amino acid protein containing 2 PWWP domains, 2 PHD-type zinc finger motifs, a TANG2 domain, an AWS domain, and a SET methyltransferase domain. The short isoform encodes a 645 amino acid protein containing only a PWWP domain. Importantly, we found that amplification and overexpression of the WHSC1L1 short isoform was predominant in a subset of aggressive breast cancers, suggesting an important role for the short isoform of the protein in breast cancer development.

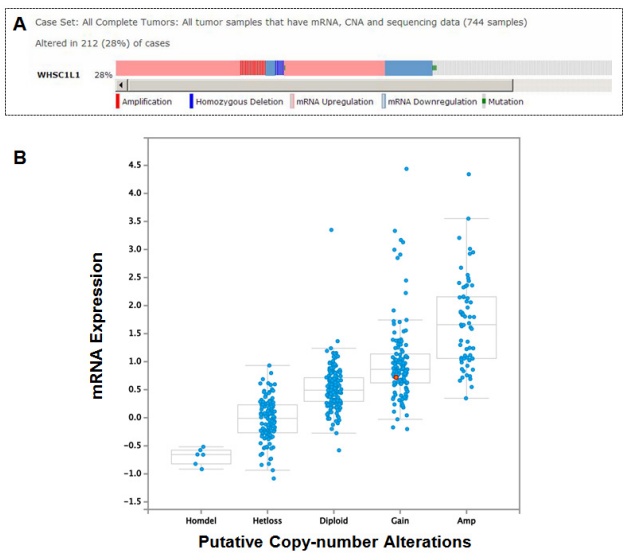

Figure 1. (A) Genomic DNA alterations and mRNA expression patterns of WHSC1L1 in 744 breast invasive carcinomas, based on the Cancer Genome database (cBio Cancer Genomics Portal). (B) WHSC1L1 mRNA expression levels are associated with DNA copy number changes in breast cancer.

Previously, we evaluated the expression of WHSC1L1 in 90 breast cancer specimens, and found that it is over-expressed in approximately 15% of specimens. Very recently, we searched the Cancer Genome Atlas database that contains 744 breast invasive carcinomas. We found DNA or mRNA alterations of

WHSC1L1 in 212 of 744 (28%) breast invasive carcinomas, where the major samples are gene amplified and/or over-expressed. We also found that WHSC1L1 mRNA expression levels are associated with DNA copy number changes in breast cancer. This new data further supports that WHSC1L1 plays an important role in breast cancer progression.

Task2. **To determine whether the histone modulation function of WHSC1L1 is linked to cancer stem cell phenotypes**.

The cancer stem cell (CSC) hypothesis suggests that only a subset of tumor cells with stem-cell-like properties is primarily responsible for the growth, progression, and recurrence of cancer (8-10). In our previous annual reports, we have reported that two *in vitro* clonogenic assays, colony formation in soft agar and mammosphere formation assay, have been used to evaluate stem-cell-like properties in MCF10-WHSC1L1 model cells. We found that MCF10A cells overexpressing WHSC1L1 short isoform grew into robust colonies in soft agar, a property not observed in the parental MCF10A cells or in MCF10A cells containing the control vector. In addition, MCF10A-WHSC1L1 cells have higher capacities to generate mammospheres rather than MCF10A control cells after 10–12 days in the mammosphere cultures. These data suggest that WHSC1L1 is likely linked to the phenotypes of cancer stem cells. However, we did not detect the mammosphere formation in the replated culture. More recently, measuring the expression of aldehyde dehydrogenase (ALDH), an enzyme previously found to be expressed in hematopoietic and neuronal stem cells, has been established as a new tool to detect normal and malignant human mammary stem cells (11, 12).. However, ALDH assays did not show direct evidence that overexpression of WHSC1L1 in MCF10A cells results in expansion of cell pools with the stem cell ALDH marker. In summary, overexpression of the WHSC1L1 short isoform likely, or at least in part, induces the acquisition of stem cell-like properties *in vitro,* but unlikely influences the self-renewal potential of breast cancer stem cells.

Task 3**. To examine the potential of WHSC1L1 as a therapeutic target in aggressive, ER-positive breast cancers that harbor the 8p11 amplicon.**

Previously, we reported that the loss-of-function approach with the lentiviral vector-based RNAi specifically targeting WHSC1L1 was performed to investigate the contribution of endogenous WHSC1L1 overexpression on the expression of transformed phenotypes in the luminal breast cancer cells with 8p11-12 amplification. We found that WHSC1L1 knock-down suppressed proliferation of WHSC1L1 amplified breast cancer cell lines, including aggressive, ER-positive SUM-44 and SUM-52 lines, while WHSC1L1 shRNAs had an undetectable effect on the cell growth of WHSC1L1 non-amplifed breast cancer cells, as well as MCF10A control cells. In addition, recently published data from the other labs also indicated that knockdown of WHSC1L1 inhibits cell growth of the 8p12 amplified, ER-positive breast cancer cells (13). Thus, our data, together with others, suggested that WHSC1L1 is a novel therapeutic target in aggressive, ER-positive breast cancers that harbor the 8p11 amplicon.

Very recently, WHSC1L1 family proteins have been shown to bind and modulate methylated histones, specifically H3K36 methylation marks (14). Therefore, we assessed global methylation (H3K4, H3K9, H3K27 and H3K36) levels by using western blotting in a panel of breast cancer cell lines, including WHSC1L1-amplified SUM-44 and SUM-52 lines. Our preliminary data indicated that global levels of H3K36me2 and me3 marks vary among different

breast cancer cell lines (Figure 2). Histone methylation levels are mediated by a large number of enzymes and regulators, including methyltransferases, demethylases and histone binding proteins. Furthermore, through the use of next-generation sequencing, it has become apparent that histone-modifying regulators, such as WHSC1L1, undergo genetic alterations at a high frequency in aggressive breast cancer. The breast cancer cell lines with defined histone methylation levels will provide a useful model for investigating biological and functional roles of these histone-modifying regulators in breast cancer, and for developing novel anticancer epidrugs.

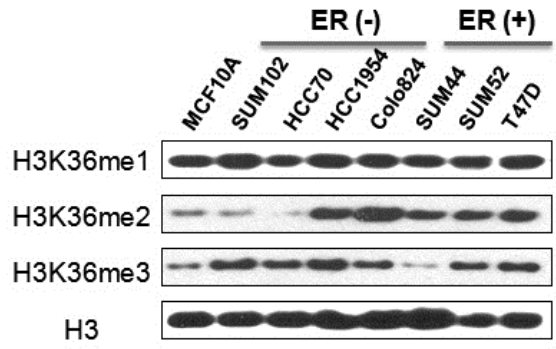

Figure 2. Global H3K36 me1, me2 and me3 levels in a panel of breast cancer cell lines. Total H3 was used as a loading control. ER: Estrogen receptor.

Remaining work for no-cost extension: In the past years, we and others found that WHSC1L1 regulates several important cancer genes that may play a critical role in breast cancer progression and development. However, we still don't know whether WHSC1L1 directly binds genomic regions of these target genes and modulates histone methylation status, due to lack of the ChIP-grade WHSC1L1 antibody. We are requesting a second no-cost extension in order to test new WHSC1L1 antibodies and determine whether inhibition of WHSC1L1 can alter histone methylation levels at the genomic regions of these WHSC1L1 candidate target genes. These experiments will provide new evidence for the establishment of WHSC1L1 as a critical epigenetic mediator for development and progression of breast cancers.

Key Research Accomplishments

Previously, we systematically investigated the transforming properties of the newly identified 8p11-12 candidate oncogene WHSC1L1 *in vitro*. We demonstrated that WHSC1L1 acts as a transforming gene: stable WHSC1L1 overexpression in nontumorigenic MCF10A cells induces transformed phenotypes, whereas WHSC1L1 knockdown in 8p12 amplified, ER-positive breast cancers cells inhibits proliferation *in vitro*. We also revealed that overexpression of WHSC1L1 likely induces the acquisition of stem cell-like properties *in vitro.* Recently, we analyzed the Cancer Genome Atlas database and revealed that DNA or mRNA alterations of WHSC1L1 in 212 of 744 (28%) breast invasive carcinomas, where the major samples are gene amplified and/or over-expressed. We also assessed global methylation levels in a panel of breast cancer cell lines, including WHSC1L1-amplified SUM-44 and SUM-52 lines. Our preliminary data indicated that global levels of H3K36 me2 and me3 marks vary among different breast cancer cell lines. The breast cancer cell lines with defined histone methylation levels will provide a useful model for studying WHSC1L1 biological and functional roles in breast cancer.

Reportable Outcomes

Presentations:

1. Yang Z-Q. Genomic alterations and oncogenic properties of histone demethylases KDM4C (GASC1) and KDM5A in human cancer, Quanticel Pharmaceuticals, San Diego, CA. Feb. 2013
2. Zhang L, Hou JL, Holowatyj A and Yang Z-Q. . The role of histone demethylase GASC1 in promoting prostate cancer progression. 104st American Association for Cancer Research Annual Meeting in Washington, DC, Apr 4-10, 2013
3. Yang Z-Q. The role of histone demethylase GASC1 in cancer and its therapeutic potential. COLD SPRING HARBOR ASIA: DifferentiationTherapy and Advances in Cancer,Suzhou, China. October 20-24, 2012 (Selected for the oral presentation)
4. Holowatyj A and Yang Z-Q. Therapeutic Potential of Histone-Modifying Enzyme GASC1 in Breast Cancer. Chicago Breast Symposium, Chicago, IL, October 13-14, 2012

Manuscript:

1. Sun LL, Holowatyj A*, Xu X, Wu J, Wu Z, Shen J, Wang S, Li E, Yang Z-Q#, Xu L. Histone demethylase GASC1--a potential prognostic and predictive marker in esophageal squamous cell carcinoma. Histopathology Submitted (# Correspondence)
2. Holowatyj A* and Yang Z-Q#. The Role of Histone Demethylase GASC1 in Cancer and its Therapeutic Potential. Current Cancer Therapy Reviews. 9:78-85, 2013 (see Appendices)

Conclusion

In the past years, we continued to investigate whether WHSC1L1 is the major driving oncogene in the 8p11-12 amplicon in a subset of breast cancer, and how over-expression of WHSC1L1 is linked to transforming and cancer stem cell phenotypes. Gain-and loss-of-function approaches provided strong evidence that WHSC1L1 possesses transforming properties, and likely plays a critical role in a subset of 8p11-12 amplified, aggressive breast cancer. The WHSC1L1 protein is involved in histone code modification and epigenetic regulation of gene expression. We have assessed global methylation levels in a panel of breast cancer cell lines, including WHSC1L1-amplified SUM-44 and SUM-52 lines. These breast cancer cell lines with defined histone methylation levels will provide a useful model for determining how WHSC1L1 contributes to its transformation through the alteration of epigenetic histone marks in breast cancer cells.

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78 *Current Cancer Therapy Reviews,* **2013**, *9*, 78-85

The Role of Histone Demethylase GASC1 in Cancer and its Therapeutic Potential

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Abstract: Interdependent genetic and epigenetic events control the initiation and progression of tumors. Genetic amplification and overexpression of the GASC1 (*gene amplified in squamous cell carcinoma 1*) gene has been found in various tumor types and this upregulation correlates with a poor prognosis for cancer patients. Gain- and loss-of –function approaches demonstrate the importance of GASC1 for the maintenance of cancer phenotypes. The GASC1 gene encodes a Jumonji C domain-containing protein, a newly identified histone lysine demethylase, that mainly catalyzes demethylation of tri- and di-methylated forms of histone H3 lysine 9 (H3K9me3/me2) epigenetic repressive marks. Recent studies indicated that over-production of GASC1 may induce alterations in epigenetic histone methylation and affects the expression of key genes that are implicated in carcinogenesis and stem cell properties in human cancer. Furthermore, histone demethylases, such as GASC1, represent highly promising anti-cancer therapeutic targets; a number of GASC1 inhibitors have been identified and reported. This review provides an overview of the current findings on genetic alterations and the biological function of GASC1 in cancer, together with a summary of recent advances in GASC1 inhibitor discovery.

Key Words: Breast cancer, esophageal cancer, GASC1, gene amplification, histone demethylase, histone methylation, therapeutic target.

INTRODUCTION

Cancer arises through the accumulation of genetic and epigenetic alterations [1]. Genetic alterations include chromosome number changes and translocations, gene amplification, deletion, and mutations; epigenetic alterations involve histone modifications, DNA methylation, and microRNA dysregulation. It is speculated that genetic and epigenetic alterations operate interdependently in the initiation and progression of cancer, e.g. epigenetic alterations can be derived from genetic alterations that dictate abnormal chromatin regulation. Recently, the use of systematic genome-wide discovery efforts has revealed the genetic alteration of histone-modifying enzymes, including histone demethylases, at a high frequency in multiple tumor types [2-6]. An imbalance between histone methylation and demethylation is believed to be implicated in tumorigenesis [7-9]. These findings highlight the central role of dysregulation of histone-modifying enzymes in tumorigenesis. Furthermore, a better understanding of the intertwined relationship between genetic and epigenetic alterations in tumorigenesis is indisputably important for the development of new prognostic markers and therapeutic targets.

In 2000, Yang *et al.* identified and cloned a novel cancer gene, called *GASC1* (*gene amplified in squamous cell carcinoma 1*), from an amplified region at 9p24 in esophageal cancer cells [2]. Later studies showed that GASC1 amplification/overexpression occurs in various tumor types, and this upregulation correlates with a poor prognosis for cancer patients [3,4,10-16]. Recently, the GASC1 protein has been identified as a member of the JMJD2 (jumonji domain containing 2) subfamily of jumonji proteins, a set of newly identified transcriptional regulators that function as histone lysine demethylases [14,17-19]. Histone demethylases play essential roles in regulating gene expression and chromatin architecture, and are thus implicated in developmental processes, aging, DNA repair, stem cell biology, and tumorigenesis [7,20-22]. Furthermore, histone demethylases, such as GASC1, represent highly promising anti-cancer therapeutic targets, not only because of their potential oncogenic roles in cancer, but also because of their druggable enzyme activities [23-26]. Here, we will review the current findings on genetic alterations of histone demethylase GASC1 (also referred to as JMJD2C or KDM4C) in multiple tumor types and discuss the potential mechanism by which GASC1 mediates epigenetic histone modifications and promotes tumorigenesis. We also highlight the recently identified GASC1 inhibitors, and discuss the potential and caveats of targeting the GASC1 demethylase for the treatment of cancer.

Identification of the GASC1 Gene from an Amplified Region at 9p24 in Esophageal Cancer

An important mechanism for the activation of oncogenes in human cancers is gene amplification, which results in gene overexpression at both the RNA and protein levels [27,28]. Yang *et al.* originally became interested in the 9p24 (GASC1) amplified region in human cancer cells after comparative genomic hybridization (CGH) analysis of esophageal cancer cell lines. Of the 29 esophageal cancer cell lines examined, 5 (17.2%) were identified that had an increase in

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Table 1. GASC1 Amplification in Various Tumor Types

Cancer Subset	Frequency of Amplification			Total Cancer Samples	Cell Lines
	Overall	Focal	High-level		
All cancers	0.115	0.0287	0.0105	3131	611
Breast	0.1564	0.0453	0.0453	243	50
Lung	0.1344	0.0478	0.0065	774	129
Esophageal squamous	0.2045	0.0227	0.0	44	12
Ovarian	0.1942	0.068	0.0194	103	7
Colorectal	0.1925	0.0062	0.0124	161	33
Glioma	0.122	0.0	0.0	41	13
Medulloblastoma	0.1328	0.0078	0.0078	128	9
Hepatocellular	0.0826	0.0165	0.0	121	11
Prostate	0.1087	0.0217	0.0	92	9
Renal	0.0238	0.0159	0.0	126	27

Note: Data was obtained from the array CGH database of 3131 cancer samples, including 611 cancer cell lines. (http://www.broadinstitute.org/tumorscape/pages/portalHome.jsf)

line KYSE150, established from a poorly differentiated, aggressive esophageal squamous cell carcinoma in a 49-year-old patient, exhibited a high level of amplification at the 9p23-24 region [2,29]. Because amplified regions often harbor oncogenes and/or other tumor-associated genes, and because 9p23-24 amplification has been reported in various other types of cancers, fluorescence *in situ* hybridization (FISH) and Southern blot analysis were used to map the 9p23-24 amplicon. Northern blotting was implemented to detect target genes/transcripts present within this amplicon, and one EST clone, R24542, was found showing overexpression in cell lines that exhibited amplification at 9p23-24. Two different cDNA libraries were screened using the R24542 clone as a probe. With this strategy, a novel gene, *GASC1*, was successfully cloned [2].

Amplification and Overexpression of GASC1 in Tumors

Recent studies clearly established that GASC1 is amplified and overexpressed in various tumor types, including lymphoma, medulloblastoma, lung, prostate and breast cancers [3,4,10-15]. Yang and colleagues performed extensive genomic analyses on a panel of breast cancer cell lines and primary samples, and found that the GASC1 region was amplified in 7 of 50 breast cancer cell lines, including HCC1954, Colo824, SUM149, HCC70, HCC38, HCC2157, and MDA-MB-436 cells; and in approximately 15% of primary breast cancers [3,15]. Based on the molecular signature, all seven GASC1-amplified lines belonged to basal-type breast cancer, an aggressive subtype of breast cancer with a poor prognosis [30]. Furthermore, by analyzing the breast cancer gene expression dataset, the level of GASC1 transcript expression was found to be significantly higher in the 116 basal-type tumors than in the 83 non-basal-type tumors (Kruskal-Wallis test $P<0.001$) [3,31]. Gain and/or amplification of the GASC1 region was also detected in approximately 35%–45% of primary mediastinal B cell lymphoma (PMBL) and approximately 33% of Hodgkin lymphoma (HL) [13]. Likewise, it is revealed that amplification of GASC1 occurs in 7.3% of medulloblastoma cases [4,32]. In another case, by combining cytogenetic, FISH, and CGH analyses of a metastatic case of lung sarcomatoid carcinoma, Italiano *et al.* detected an amplification of the GASC1 region and showed that this amplification was a significant element for pathogenesis of this tumor because it was detected in two different metastases as well as in the primary tumor [11].

To further demonstrate that the GASC1 gene is amplified in various tumor specimens, we queried the array CGH database: a collection of 3131 copy-number profiles across multiple cancer types [33]. In these 3131 tumor samples, there are 11.5% cases containing GASC1 amplification, where the GASC1 gene is also in the focal amplification peak in 2.87 % cases, particularly in breast and lung cancers (Table **1**). In 243 breast cancer samples, there exist 15.64% cases containing GASC1 amplification, in which 4.53% cases have the high-level amplification based on the GISTIC (Genomic Identification of Significant Targets in Cancer) analysis. In 774 lung cancer samples, there are 13.44% cases containing GASC1 amplification. In esophageal squamous cancer samples there are 20.45% exhibiting amplification, for ovarian cancer 19.42%, and for colorectal cancer 19.25% (Table **1**). It has also been reported that the expression of GASC1 is significantly increased in prostate cancers relative to normal tissue [14,16]. In summary, GASC1 is amplified and overexpressed in multiple tumor types.

Transforming Properties of GASC1

Since the discovery of the *GASC1* gene, studies have shown its transforming properties in various cell models. To test whether GASC1 is potently transforming in human mammary epithelial cells, wild-type GASC1 was cloned into a lentiviral vector and transduced into human nontumorigenic mammary epithelial MCF10A cells. Over expression of GASC1 in MCF10A cells resulted in the acquisition of phenotypes that are hallmarks of neoplastic transformation, including growth factor-independent proliferation and anchorage-independent growth in soft agar [3]. To further examine the effects of GASC1 activity in a context that more closely resembles *in vivo* mammary architecture, Yang and

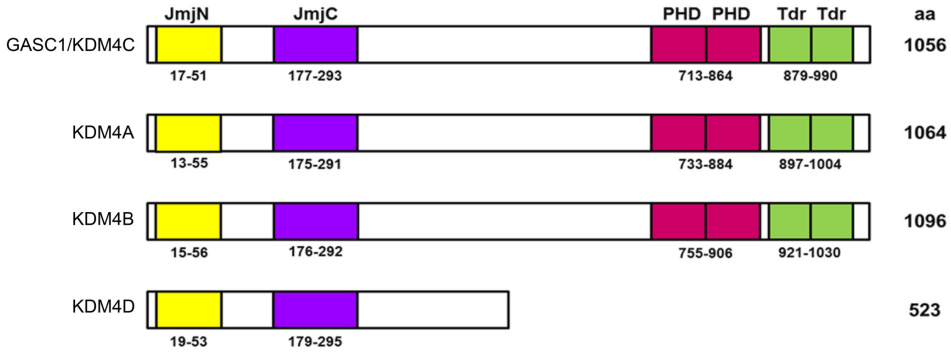


Fig. (1). Domain structure of GASC1 and its homologues KDM4A, B and D. The location and length of each domain is based on the data from the National Center for Biotechnology Information (NCBI).

pression on three dimensional morphogenesis in Matrigel. Whereas MCF10A cells formed polarized, growth-arrested acinar structures with hollow lumens similar to the glandular architecture *in vivo*, MCF10A-GASC1 cells formed abnormal acini at a high frequency that were grossly disorganized, and contained filled lumens [3]. These results indicate that GASC1 over expression disrupts epithelial cell architecture, which occurs frequently during the early stages of cancer formation.

The importance of GASC1 for the maintenance of cancer phenotypes has also been shown in breast, esophageal, prostate cancers and lymphoma with shRNA knockdown approaches. The Expression Arrest GIPZ lentiviral shRNAmir system was used to stably knock down GASC1 expression in three GASC1 amplified breast cancer cell lines, HCC1954, Colo824 and SUM149, demonstrating that GASC1 inhibition significantly slowed cell growth and inhibited colony formation of GASC1-amplified breast cancer, while it had only a slight effect on the cell growth of MCF10A control cells [3]. Inhibition of GASC1 expression caused a significant reduction of proliferation in the KYSE150 esophageal cancer line [14]. In prostate cancer, it was reported that the GASC1 protein interacts with the androgen receptor (AR) and functions as a co-activator of AR-induced transcription; reduction of GASC1 with shRNAs inhibited androgen-dependent proliferation of prostate cancer cells [34]. As mentioned above, the 9p24 region is frequently amplified in lymphomas, specifically PMBL and HL. To identify oncogenes in this amplicon, Rui *et al.* employed an unbiased approach using RNA interference genetic screening to discover the functionally critical genes in the 9p24 amplicon in PMBL and HL. They found that two genes, GASC1 and JAK2, cooperate to sustain the proliferation and survival of these lymphomas [13]. In summary, evidence has accumulated indicating the oncogenic roles of GASC1 in several types of cancer cells.

GASC1 as a Histone Demethylase

Chromatin modification has emerged in the last few years as an important mechanism of epigenetic regulation; it is clear that aberrant regulation of histone modification is relevant to the initiation and progression of cancer [1,9]. The basic unit of chromatin is the nucleosome that consists of 147 base pairs of DNA wrapped around a repetitive nucleosome core composed of four couples of histones H2A, H2B, H3 and H4. These histones are predominantly globular except for their N-terminal tails, which contain a plethora of posttranslational modifications. Histone tail modifications include methylation, acetylation, phosphorylation, ubiquitination and isomerization, resulting in a combination of histone marks referred to as the histone code [35-37]. Histone lysine methylation, governed by the opposing activities of histone methyltransferases and demethylases, serves as the principal chromatin-regulatory mechanism that influences fundamental nuclear processes and has a central role in transcriptional regulation [21,22,35]. Different transcriptional and biological outcomes result from methylation at different lysine residues, degree of methylation at the same lysine residues, and the location of the methylated histone within a specific gene locus. Lysine methylation at five sites on histone H3 (K4, K9, K27, K36, and K79) has shown an effect on gene transcription [21,22,35]. In general, methylation of H3K4, H3K36 and H3K79 are associated with the activation of transcription, whereas tri- and di-methylated forms of H3K9 (H3K9me3/me2) and H3K27 (H3K27me3/me2) are associated with repression of transcription [38,39].

When GASC1 was originally cloned in 2000, it was predicted that GASC1 is likely a nuclear protein involved in chromatin-mediated transcriptional regulation; however, at that time, the role and mechanism of this protein that regulates cellular processes, including transcriptional regulation in normal and cancer cells, was unknown. In 2004, Katoh *et al.* determined that GASC1 belongs to the JMJD2 (Jumonji domain containing 2) subfamily of the Jumonji family [40]. This group designated GASC1 as JMJD2C which contains one Jumonji (Jmj)C domain, one JmjN domain, two Plant Homeo Domain (PHD)-type zinc fingers and two Tudor domains (Fig. **1**). In 2006, there was a breakthrough in the understanding of how chromatin is regulated with the identification of JmjC domain-containing proteins, including GASC1, as a new class of histone demethylases [14,18,41]. In 2007, the new name KDM4C (lysine-specific demethylase 4C) was given to the GASC1 protein [42]. On the basis of homology, the JmjC family consists of 30 members, and thus far 18 of these have been identified to possess histone demethylase activity, and were further classified into seven subfamilies (KDM2-8) [21]. There are six members (KDM4A-F) of the human KDM4 (JMJD2) subfamily, of which two, KDM4E/F, are likely to be pseudogenes [40]. The KDM4A, B and C (GASC1) proteins, that share more than 50% percent of sequence identity, contain JmjN, JmjC,

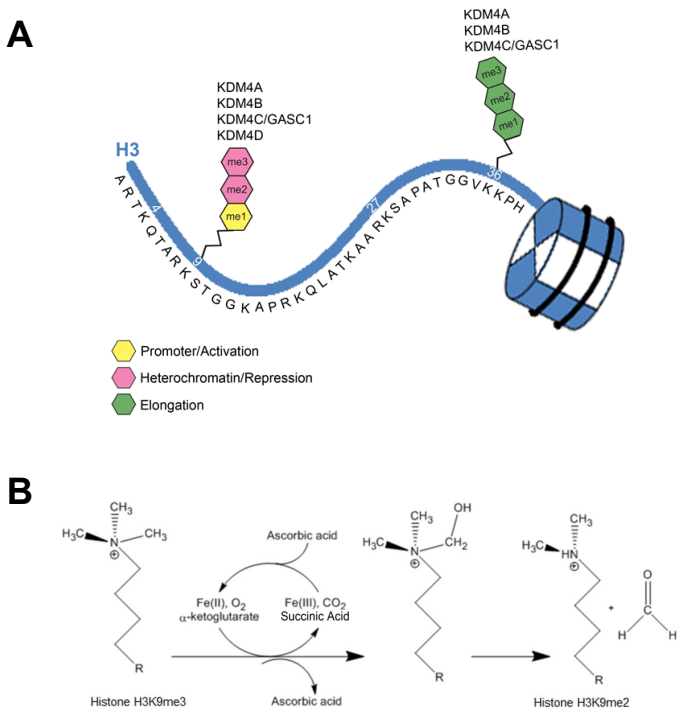


Fig. (2). GASC1 functions as a histone demethylase. (A) GASC1 catalyzes demethylation of H3K9me3/me2 and H3K36me3/me2. In general, H3K9me3/me2 is associated with repression of transcription while methylation of H3K36 is associated with transcriptional elongation. (B) GASC1 catalyzes the hydroxylation of methyllysine residues in a Fe(II) and α-ketoglutarate-dependent manner, releasing succinate and CO_2. In a second, nonenzymatic step, formaldehyde is spontaneously released after the decomposition of the N-hydroxymethyl moiety. For simplicity, only trimethylated H3K9 is illustrated. The same mechanism can be applied for demethylation of di-, or trimethylated H3K9 and H3K36.

PHD and Tudor domains, while the KDM4D protein lacks C-terminal PHD and Tudor domains (Fig. **1**).

The N-terminal Jumonji domain of GASC1 is the histone demethylase catalytic core [14,18,41]. Jumonji, which is Japanese for "cruciform", was the name given to the transcription factor whose ablation in mice resulted in neural plate deformation that resembles a cross [43]. Two conserved sequences have been noted in Jumonji and were referred to as JmjN and JmjC based on their relative locations to each other within the protein [44-46]. The JmjC domain is the catalytic domain of GASC1 for histone demethylation; the JmjN domain has been found to provide structural integrity and to form extensive interactions with the catalytic JmjC domain [17,19,41]. Both JmjC and JmjN are essential for the demethylase activity of JmjC-containing-proteins including GASC1 [17,47]. The demethylase reaction catalyzed by the Jumonji domain of GASC1 is a dioxygenase reaction that depends on two cofactors, Fe(II) and α-ketoglutarate (α-KG) (Fig. **2**). Early studies demonstrated that the Jumonji domain of GASC1 catalyzes demethylation of H3K9me3/me2 and H3K36me3/me2 *in vitro* and in cells [14,17-19]. Recently, combined structural, biochemical, and cellular studies demonstrated that GASC1 catalyzes the demethylation of H3K9me3/me2, and less efficiently (4-5 fold less than H3K9me3/me2), H3K36me3/me2 substrates [48]. In addition, competitive experiments employing H3K9me3 and H3K9me2 peptides revealed a clear preference for the tri- over the dimethylated state for the GASC1 substrate [48]. The crystal structure of the GASC1 catalytic domain (1-347 aa) is available at the Protein Data Bank (PDB) database (2XML). *In vitro* biochemical and cellular assays revealed that residues H190, E192 and H288 within the JmjC domain of GASC1 form an essential part of the Fe(II)-binding groove, and mutating H190 and E192 is sufficient to abrogate its H3K9 demethylation activity [14]. Thus, multiple studies indicate that the Jumonji domain of GASC1 predominantly demethylates H3K9me3. On the other hand, C-terminal PHD and Tudor domains likely contribute to efficient nuclear localization of GASC1 and mediate GASC1 associating with histone and other proteins [49]. However, the exact roles of the GASC1 PHD and Tudor domains in GASC1-dependent chromatin regulation remain unclear.

Molecular Mechanisms and Potential Functions of GASC1 in Tumorigenesis

The importance of H3K9 and H3K36 di- and tri-methyl marks in transcription and other processes that GASC1 regulates implies that the regulation of GASC1 is necessary and important for normal cellular function. Thus, it is speculated that deregulation of GASC1 can lead to imbalances in histone methylation pathways that affect many chromatin-regulated processes, of which transcription regulation, DNA repair, and chromosome stability are the most relevant for the pathogenesis of human cancers. Several recent studies provide clues on the mechanisms by which GASC1 contributes to tumorigenesis via its histone demethylation function. In general, H3K9me3/me2 marks are associated with the promoter of silenced euchromatic genes; its removal by

GASC1 will result in transcriptional activation [38,39]. Indeed, studies have shown that GASC1 enhances the expression of important genes, such as classical oncogenes MDM2 and MYC, as well as key stem transcription factor NANOG, through its H3K9 demethylation function [3,13,50-52]. In order to uncover the function of GASC1 in oncogenesis, Ishimura *et al.* searched for the downstream target genes regulated by GASC1 using mouse embryonic fibroblasts (MEFs) [50]. Exogenous overexpressing GASC1 in MEF cells increases the expression of the MDM2 oncogene at the mRNA and protein levels. A chromatin immunoprecipitation (ChIP) assay showed that GASC1 was recruited to the P2 promoter region of the MDM2 gene, resulting in demethylation of H3K9me3/me2. However, there was no detectable change of the H3K36me3 level at the P2 promoter of MDM2 with GASC1 overexpression. Furthermore, siRNA-mediated knockdown of GASC1 caused reduction of MDM2 expression in the cells. Wissmann *et al.* identified GASC1 as the first histone tri-demethylase regulating AR function [34]. GASC1 interacts with the AR *in vitro* and *in vivo*; assembly of ligand-bound AR and GASC1 on the promoter of AR-target genes results in demethylation of H3K9me3 and stimulation of androgen receptor-dependent transcription. Conversely, knockdown of GASC1 inhibits androgen-induced removal of H3K9me3 and transcriptional activation. Rui *et al.* demonstrated that knockdown of GASC1 by shRNA inhibits MYC expression by directly altering the H3K9me3 mark at the promoter and intron 1 regions of MYC in lymphoma cells.

The cancer stem cell (CSC) hypothesis suggests that a subset of tumor cells with stem-cell-like properties is primarily responsible for the growth, progression and recurrence of cancer [53-55]. H3K9 methylation status in pluripotent embryonic stem cells (ESCs) is maintained both globally and locally by an intricate interplay between the activities of pluripotency factors and histone demethylases [52,56,57]. Functionally, key pluripotent factors, including OCT4 (POU5F1), NANOG and SOX2, form a robust autoregulatory circuit that maintains ESCs in a self-renewing state [57,58]. Interestingly, GASC1 is preferentially expressed in undifferentiated ES cells [59]. In 2007, Loh *et al.* identified GASC1 as a *bona fide* target of the OCT4 in mouse ESCs [52]. More significantly, they identified NANOG as a target of GASC1 and confirmed the recruitment of GASC1 to the NANOG promoter [52]. They demonstrated that GASC1 is required to reverse H3K9me3 repressive marks at the NANOG promoter region in ESCs. Loss-of-function approaches illuminated that GASC1 is critical for the maintenance of the self-renewal state of ES cells [52]. Thus, GASC1 is a component of the ESC transcription circuitry designed to maintain ESC properties. Notably, introduction of GASC1 in MCF10A cells could increase higher capacity to generate mammospheres, a phenotype of cancer stem cells [3]. Because of its regulation of important ESC factors, GASC1 could provide a link between stem cell function and cancer initiation and progression when it functions as an oncogene. The effects of GASC1 demethylase function on cancer stem cells are still under intense investigation, and many questions remain largely unanswered. Nevertheless, recent studies support the notion that amplification and subsequent over-production of GASC1 induces alterations in epigenetic histone methylation epigenetic histone methylation and affects the expression of a set of key genes that are implicated in carcinogenesis and stem cell properties in human cancer.

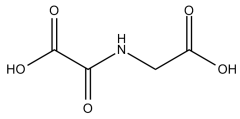
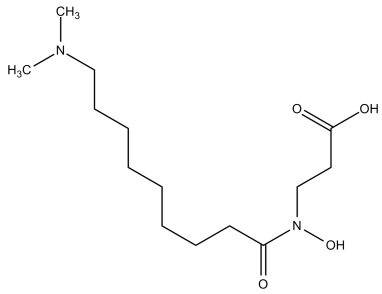
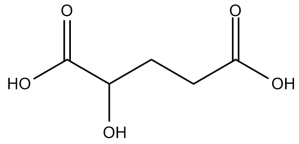
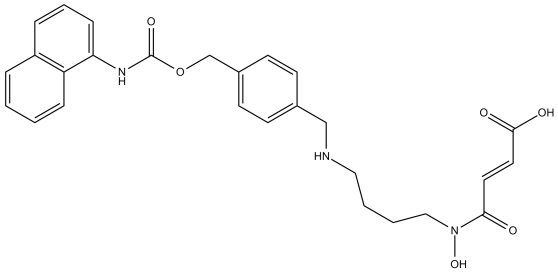
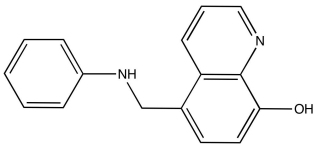
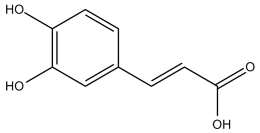
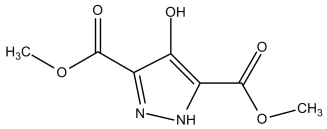
GASC1 as a Potential Therapeutic Target

The discovery that histone demethylases, including GASC1, play critical roles in tumorigenesis by controlling epigenetic oncogenic programming provides a unique opportunity to develop demethylase inhibitors as a novel class of anti-cancer drugs [23,24,60,61]. On the basis of the three–dimensional structure and catalytic mechanism of the GASC1/JMJD2 family of histone demethylases mentioned above, a number of JMJD2 inhibitors have been identified and reported. Here, we focus on the reported inhibitors of GASC1 (Table **2**).

The GASC1 demethylase is an Fe(II)- and α-KG-dependent enzyme that oxygenates methylated histone lysine residues, which in turn leads to their demethylation (Fig. **2**). The efforts to target cofactors essential for the activity of GASC1 has provided the first promising results. N-oxalylglycine (NOG), first tested by Cloos *et al.* in 2006, was found to weakly inhibit the GASC1 demethylation of H3K9me3 [14]. As an α-KG analogue, it is speculated that NOG displaces α-KG from the iron-binding residues of GASC1, inhibiting GASC1 activity. Based on the crystal structure model of the GASC1 Jumonji domain complexed with α-KG, Hamada *et al.* designed and synthesized a series of GASC1 small molecule inhibitors. Compound 8 (later named NCDM-32), was found to be the most selective and potent GASC1 inhibitor [26,62]. Compound 8, with eight methylene chains, showed a low micromolar IC_{50} value against GASC1 as compared to NOG (Table **2**). The noted interactions between GASC1 and Compound 8 indicate the importance in potency for the tertiary amino group as well as the linker length of the inhibitor for interaction. Overall, Compound 8 showed 500-fold greater GASC1-inhibitory activity than NOG [62]. By using biochemical, structural and cellular assays, Chowdhury *et al.* found that 2-hydroxyglutarate (2HG) inhibits α-KG-dependent oxygenases, including GASC1 [63]. Methylstat, a cell-active selective histone demethylase inhibitor, inhibits the subfamily of trimethyl lysine demethylases. This small molecule inhibitor contains a (methyllysine) substrate mimic, an (α-KG) cofactor mimic, and a linker to attach them (Table **2**). Importantly, Methylstat inhibited cell growth of GASC1 amplified KYSE150 esophageal cancer with a half maximal growth inhibitory concentration (GI_{50}) at approximately 5.1 μM.

A high-throughput RapidFire mass spectrometry assay was used to screen more than 100,000 compounds to identify GASC1 inhibitor candidates. This assay employs a short amino acid peptide substrate, corresponding to the first 15 amino acid residues of histone H3, and monitors the direct formation of the dimethylated-Lys9 product from the trimethylated-Lys9 peptide substrate [64]. With this assay, 1126 compounds have been found with IC_{50} values less than 100μM. For example, Compound 5, that contains the core structure of 8-hydroxyquinolines (8HQs), displayed strong potential (IC_{50} : 2.1 μM) to inhibit GASC1 demethylase activity. Another study demonstrated that 8HQs inhibit KDM4

Table 2. Summary of GASC1 Small Molecular Inhibitor

Name	Structure	Activity (IC_{50})	References	PubMed ID Numbers
N-oxalylglycine (NOG)		500 μM	Cloos *et al.* (2006)	16732293
Compound 8 (NCDM-32)		1.0 μM	Hamada *et al.* (2010) Suzuki *et al.* (2011)	20684604 21955276
2-hydroxyglutarate (2HG)		(*R*)-2HG = 79 μM (*S*)-2HG = 97 μM	Chowdhury *et al.* (2011)	21460794
Methylstat		3.4 μM	Luo *et al.* (2011)	21585201
Compound 5		2.1μM	Hutchinson *et al.* (2012)	21859681
Compound 1 (Caffeic Acid)		13.7 μM	Nielsen *et al.* (2012)	22575654
Compound 2		147 μM	Leurs *et al.* (2012)	22917519

subfamily demethylases via binding to the active-site Fe(II) and display activity against KDM4A in cell-based studies [65]. Upon screening a 640 member natural product library for inhibitors of GASC1, Nielsen *et al.* tested a subset of 21 compounds in the formaldehyde dehydrogenase assay and discovered Compound 1 (Caffeic Acid) as a GASC1 inhibitor (Table **2**). Compound 1 is a known anti-oxidant shown to inhibit cancer cell proliferation through oxidative processes [66]. Very recently, a heterocyclic ring system library was screened against GASC1 in the search for novel inhibitory scaffolds. A 4-hydroxypyrazole scaffold (Compound 2) was identified as a new inhibitor of KDM4C (Table **2**) [67].

PERSPECTIVES AND CONCLUSION

Cancer is traditionally viewed as a genetic disorder; however, accumulated evidence shows that epigenetic disruption plays a critical role at every stage of tumorigenesis and holds a significant impact on tumorigenic mechanisms and the development of cancer therapies. While epigenetics and genetics can cooperate in cancer initiation and progression, the interconnectedness between these two processes is becoming increasingly apparent with the realization that epigenetic modifiers are genetically altered at a high frequency in multiple tumor types. Notably, the GASC1 gene was originally discovered and cloned from the genetic amplified region in esophageal cancer. Lately, it has been identified as the key epigenetic histone modifier, histone lysine demethylase, which plays an important role in epigenetic histone modification. Recent studies revealed that GASC1 is amplified and over-expressed in various aggressive tumors, and is implicated in the transforming phenotypes in several *in vitro* models. However, many vital questions remain regarding the molecular mechanisms by which GASC1-dependent chromatin regulation translates into oncogenicity and contributes to cancer initiation and progression. For example, to better understand how GASC1 affects chromatin organization and transcription, it will be critical to determine the genome-wide targets of GASC1, as well as the effect of GASC1 deletion and overexpression on transcription and histone modification patterns. It is important to investigate whether GASC1 targets different genes in different types of cancer. It also should be noted that GASC1 has been reported to demethylate non-histone substrates *in vitro* and *in vivo*, and the identified substrates share sequence similarity to H3K9 [68-70]. Very recently, it is revealed that GASC1 can demethylate the K191me2 of the chromobox homolog 4 (CBX4, also known as polycomb 2 protein: Pc2), which plays an important role in cell cycle and growth control [70]. However, the interplay between histone and non-histone methylations regulated by GASC1 has not been addressed. Thus, increasing an understanding of this exciting biology and the mechanisms of GASC1 demethylation function is a significant component of further studies and research.

Given the critical roles of GASC1 in cancers, it is very likely that inhibitors of GASC1 will move forward into clinical trials. However, one must keep in mind the caveat that most GASC1 inhibitor scaffolds derive from other structurally or mechanistically related enzymes and these compounds are, therefore, oftentimes also active against other enzyme families. In addition, most inhibitors are cofactors and/or substrate mimics and so far have only very limited or undetermined specificity for GASC1. It will, thus, be an utmost objective for the near future to discover more potent and, especially important, more selective inhibitors with the ability to specifically target GASC1.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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